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(54) Title: METHOD OF DETECTING COMPOUNDS UTILIZING GENETICALLY MODIFIED LAMBDOID BACTERIOPHAGE

(57) Abstract

Disclosed is an effective lambdoid bacteriophage which includes a protein construct comprising a genetically modified major tail protein truncated at its carboxy terminus, and a target molecule peptide bonded to the carboxy terminus of the tail protein. Also disclosed are nucleic acids encoding the construct and methods of detecting a molecule-of-interest in a solution and of detecting a cell which produces a molecule-of-interest.

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METHOD OF DETECTING COMPOUNDS UTILIZING GENETICALLY MODIFIED LAMBDOID BACTERIOPHAGE

FIELD OF THE INVENTION

This invention relates to the detection of compounds, and more specifically to methods for detecting and assaying for a molecule-of-interest and for cells producing such a molecule-of-interest utilizing a genetically modified lambdoid bacteriophage.

BACKGROUND OF THE INVENTION

Bacteriophages have been used in strategies for detecting molecules-of-interest. For example, a method employing the bacteriophage M13 has been used to assay for various proteins of interest. In this method, M13 phage displaying peptides fused to pIII, a minor M13 coat protein, have been used to screen for protein binding molecules and antibodies (Scott et al. (1990) *Science* **249**:386; Devlin et al. (1990) *Science* **249**:404). Special M13-derived systems have been used to express antibodies as fusion proteins on the surface of the phage, and techniques have been developed to enrich the population for phage expressing antibodies with desired affinities for an antigen (Garrard et al. (1991) *Bio/Technol.* **9**:1373; Barbas et al. (1991) *Proc. Natl. Acad. Sci.* (USA) **88**:7978). However, the use of M13 in assay methods is limited because M13 infection is not immediately ascertainable. This is because infection by M13

does not provide the cell with compounds required for growth and is not lytic.

Like M13, T4 has been used in assays for various proteins such as nerve growth factor (NGF) (Olger et al. (1974) *Proc. Natl. Acad. Sci.* (USA) **71**:1554-1558). In this assay, T4 was chemically coupled to NGF using glutaraldehyde. The phage was then rendered non-infective by treatment with antibodies against NGF. When unbound NGF was added to the medium, NGF-linked phage was displaced from the antibody and became free to infect *Escherichia coli (E. coli)*. Bacteriophage T4 has also been used to detect antibodies against a wide range of compounds. For example, Becker et al. (*Immunochem.* (1970) **7**:741) used a T4 bacteriophage to detect antibodies against p-azobenzenearsonate. Hurwitz et al. (*Eur. J. Biochem.* (1970) **17**:273) used a T4 bacteriophage to detect and estimate levels of angiotensin-II-beta-amide and its antibodies. Gurari et al. (*Eur. J. Biochem. (1972)* **26**:247) used bacteriophage T4 in the detection of antibodies to nucleic acids. These detection methods involve the chemical modification of the T4 phage resulting in the non-specific exposure on the phage surface of a compound to which the antibodies to be assayed are targeted. Such antibodies render the bacteriophage non-infective, thus enabling the decrease in plaque formation to be used as a measure of the level of antibody present. The T4 system has also been used to measure hapten concentrations (see, e.g., Hurwitz et al. (1970) *Eur. J. Biochem.* **17**:273-277) In this system, T4 is chemically

modified such that it exposes the desired hapten non-specifically on its surface. The addition of anti-hapten antibody destroys the infectivity of the phage. Infectivity is restored in the presence of hapten.

Although both the M13 and T4 phage systems can be used to detect the presence of a compound by their ability to become infectious in the presence of that compound, infection by M13 is normally not immediately ascertainable, and T4 infection is lethal. Thus, these systems cannot be used where a quick screening or selection method based on the survival of the infected bacterial cell is desired, such as where a particular cell type is being selected, or when the object of phage infection is to restore the ability of an auxotrophic bacterial cell to survive on its own under a given set of growth conditions. Special M13-derived phagemid systems carry genes which could endow an infected cell with a selective growth advantage (Barbas et al. (1991) *Proc. Natl. Acad Sci.* (USA) 88:7978). However, these systems have not been used to detect a molecule-of-interest or cells producing such compounds. Furthermore, because gpIII, the M13 protein to which the target molecules are fused, accumulates on the inner membrane facing the periplasm, there are limitations on the nature of the protein fusion. Fusions that are not able to cross the membrane will not be assembled into M13. In addition, in all M13 systems where fusion proteins have been used to display proteins on the

outer surface, the displayed protein (or peptide) itself has been the molecule-of-interest.

Thus, what is needed are methods for assaying for molecules-of-interest and for cells producing such molecules which are efficient, accurate, and fast. What are also needed are assay methods which do not have to result in bacterial cell death. Additionally, assay methods utilizing bacteriophage infection are needed for non-proteinous molecules of interest and for cells which continuously produce these molecules-of-interest.

SUMMARY OF THE INVENTION

It has been previously determined that removal of up to one third of the gpV protein of the bacteriophage lambda does not affect the assembly or infectivity of the phage (Katsura (1981) *J. Mol. Biol.* **146**:493-512). Furthermore, it has been discovered that lambdoid bacteriophage having a target molecule peptide linked to one of its components, the gpV protein, can be successfully assembled *in vivo* such that the target molecule is displayed on the outer surface of the phage. In addition, the genetically modified lambdoid bacteriophage maintains its ability to infect *E. coli*. These findings have been exploited to develop the present invention, namely, methods of detecting a molecule-of-interest in a solution and of detecting a cell which produces such a molecule-of-interest, utilizing a genetically modified lambdoid bacteriophage.

As used herein, the term "lambdoid bacteriophage" is meant to encompass all lambda-related phages and all derivatives, genetically engineered derivatives, and hybrids thereof, such as, but not limited to, Φ80, Φ81, phages 21, 82, 424, 432, λimm434, λimm21, phagemids, λEMBL, and λgt.

In this method, a protein construct is provided which includes a genetically modified gpV protein truncated at its carboxy terminus and a target molecule peptide bonded to the carboxy terminus of the truncated gpV protein. As used herein the term "gpV protein" is meant to encompass any major tail protein found in the lambdoid bacteriophages. This includes but is not limited to lambda gpV protein, gpV-related proteins and equivalents of lambda gpV protein in the tails of other lambdoid viruses. In preferred embodiments of the invention, the target molecule is a protein such as an enzyme, enzyme substrate, immunoglobulin, or binding fragment thereon, hormone, ligand, toxin, growth factor, cytokine, receptor, or a fragment or analog of any such protein.

In some embodiments the protein construct further includes at least an antigenic portion of a third protein, or fragment thereof, to which antibodies have been raised. A preferred third protein is a marker protein such as ß-galactosidase, chloramphenicol acetyltransferase, or alkaline phosphatase. As used herein, the term "marker

protein" refers to the protein or fragment thereof to which an antibody is available.

In one aspect of the invention, the protein construct is provided by transforming a bacterial cell with a nucleic acid encoding the protein construct. This bacterial cell has been preinfected with a lambdoid bacteriophage assembly mutant that has defective or substantially no gpV protein. The transformed cell is induced to express lambdoid components and the protein construct, and then to assemble a lambdoid phage therefrom, the phage having the target protein on its outer surface. The bacteriophage are then isolated from the cell.

In another embodiment the lambdoid bacteriophage is provided for use in the method of the invention as follows. A bacterial cell is infected with a lambdoid bacteriophage assembly mutant having defective or absent gpV protein. This bacterial cell has been pre-transformed with a nucleic acid encoding the protein construct. The cell is induced to express the viral components and protein construct and to assemble a lambdoid phage therefrom. The lambdoid phage thus formed has the target protein on its outer surface.

The target molecule on the bacteriophage is then processed such that the phage is rendered reversibly non-infective or inactive, (i.e., with further treatment the non-infective phage can become infective again). In some aspects of the invention, inactivation is accomplished by treating the

bacteriophage with a molecule that binds the target molecule. The binding of the target molecule renders the phage non-infective. Preferably, the binding molecule is an immunoglobulin, or binding portion thereof, specific for an antigenic determinant on the target molecule, a receptor specific for a ligand-type target molecule, or an immobilized ligand which binds to a receptor-type target molecule. In other aspects, the binding molecule is a matrix to which the bacteriophage-linked target molecule is immobilized. Immobilization renders the phage non-infective because it cannot bind to the lambda cell receptor.

The non-infective bacteriophage is then treated with a solution which contains a molecule-of-interest. In some preferred embodiments the solution is a cell lysate, cell culture medium, or a biological sample such as blood, urine, saliva, serum, semen, or lacrimal secretions.

The term "molecule-of-interest" is meant to encompass any molecule whose activity or presence is desired, and which can render the non-infective bacteriophage infective again. Useful molecules-of-interest are proteins, peptides, hormones, nucleic acids, carbohydrates, lipids, glycoproteins, glycolipids, proteolipids, lipoproteins, lipopolysaccharides, vitamins, toxins, terpenes, antibiotics, and cofactors.

In some embodiments, the molecule-of-interest is a protein such as an enzyme which cleaves the

target molecule, an enzyme substrate. Cleaving of the binding molecule-linked target molecule liberates the bacteriophage from the binding molecule, thereby rendering it infective once again.

In other embodiments, the molecule-of-interest is unbound target molecule. Unbound target molecules present in the solution-to-be-tested displace the binding molecule on the phage-linked target molecule and bind with the binding molecule, thereby liberating the phage and rendering it infective once again. In another aspect of the invention, the molecule-of-interest is different than the target molecule but yet is capable of binding to the binding molecule, thus displacing the target molecule.

In one preferred embodiment, the target molecule and the molecule-of-interest are the same and are ligands, and the binding molecule is a receptor specific for that ligand. In another embodiment, the target molecule and the molecule-of-interest are the same and are receptors, and the binding molecule is a ligand that binds that receptor. In yet another embodiment, the target molecule and the desired molecule (or molecule-of-interest) contain the same antigenic determinant, and the binding molecule is an immunoglobulin, or portion thereof, that binds to that antigenic determinant. In still another embodiment, the target molecule and the molecule-of-interest are the same and are immunoglobulins, or binding portions

thereof, and the binding molecule contains an antigenic determinant bound by that immunoglobulin.

In the method of the invention, a bacterial cell such as an *E. coli* cell, is contacted with the treated bacteriophage for a time sufficient for the bacteriophage to infect the cell. The infected cells are then detected, infection being indicative of the presence of the molecule-of-interest in the solution which has rendered the bacteriophage infective.

In some embodiments, detection is accomplished by observing cell death in the form of cell lysis or plaque formation. Lysis results when the nucleic acid of the phage successfully enters the cytoplasm of the cell, directs the cell to produce viral components at the expense of cellular components and to assemble them into phage particles, and causes the cell to rupture or lyse such that the assembled viral particles are released. Plaques result when multiple neighboring cells plated on solid culture dishes lyse in this way, leaving clear or empty spots on the otherwise cloudy culture lawn.

In other aspects of the invention, detection of infection is accomplished by observing bacterial cell survival and/or growth at or below 32°C where the bacterial cell infected by the phage is an auxotrophic mutant requiring a gene supplied by the phage for survival and growth and where the phage is a temperate, temperature sensitive phage. In this aspect, the phage, once rendered infective again,

infects a bacterial cell by injecting its nucleic acid into the host cell.

As used herein, the term "temperate phage" refers to a phage that can be lytic or lysogenic. When lysogenic, the phage integrates its nucleic acid into the host cell genome and remains quiescent, replicating only when the host genome replicates. In its lytic or vegetative multiplication phage, the phage nucleic acid excises itself from the host genome, or does not integrate itself into the host cell genome, but rather takes over the protein synthetic machinery of the cell at the expense of cellular components and causes phage progeny to be assembled. New phage are released from the cell when the cell lyses. A temperate phage may contain a mutation conferring temperature sensitivity, i.e., it is lysogenic only at low growth temperatures (e.g., at or below about 32°C) and is lytic at high growth temperatures (e.g., at about 37°C and above, such as at about 42°C). Thus, at lower growth temperatures, the lysogenic phage DNA integrates into the bacterial cell genome, providing the genome with a gene which the auxotrophic cell requires to survive. Preferably, such a gene encodes a needed protein.

In another embodiment, detection of infection is also accomplished by observing bacterial cell survival and/or growth in those embodiments of the invention where the phage, which is temperature sensitive as described in the above paragraph, carries a gene encoding antibiotic resistance.

Infection of *E. coli* by this phage will permit the former to survive/grow on media containing the antibiotic whose resistance is encoded by the gene carried by the phage.

In some embodiments, cells that secrete/excrete the molecule-of-interest can be selected from a generally non-secreting population. In these embodiments, bacterial cell growth is indicative of phage infection, and hence, of the secretion/excretion of the molecule-of-interest. The bacterial cell to be infected is an auxotroph which itself produces and secretes the molecule-of-interest, which is the same as the target molecule and thus is capable of displacing the target molecule from the binding molecule. In this method the phage carries a bacterial gene encoding a protein required by the auxotrophic bacterial cell for survival. The phage is inactivated by antibodies directed to the target molecule, and then is contacted with the solution-to-be-tested which may be medium in which the mutant bacterial cell had been growing and/or with the bacterial cell, itself. If the medium contains unbound molecule-of-interest, or if the cell is producing and secreting it, antibody bound to the phage linked target molecule is displaced and instead binds to the unbound molecule-of-interest in the solution. The liberated phage then infects the bacterial cell, and at lower growth temperatures (e.g., at or below about 32°C), provides the cell with the bacterial gene it needs for growth.

The invention also includes the protein construct described above, nucleic acids or gene fusions encoding those protein constructs, and genetically modified, infective lambdoid bacteriophage displaying the target molecule on their outer surface.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1A is a diagrammatic representation of the bacteriophage lambda;

FIG. 1B is a diagrammatic representation of the genetically modified bacteriophage lambda of the invention;

FIG. 2 is a schematic representation of the nucleic acid sequence and corresponding amino acid sequence of the gpV protein;

FIG. 3 is a schematic representation of the strategy for constructing the truncated V gene with a multiple cloning site at its carboxy terminus;

FIG. 4 is a schematic representation of the 3' and 5' primers used to provide the PCR fragment containing the full length, modified V gene in plasmid pSYM1;

FIG. 5A is a schematic representation of the pSYM1 plasmid containing the PCR fragment of FIG. 4;

FIG. 5B is a schematic representation of plasmid pSYM2 containing a truncated V gene with multiple cloning sites;

FIG. 5C is a schematic representation of plasmid pSYM3 containing a truncated V gene and a gene encoding a marker protein;

FIG. 6A is a diagrammatic illustration of one embodiment of the method of the invention;

FIG. 6B is a diagrammatic illustration of another embodiment of the method of the invention;

FIG. 6C is a diagrammatic illustration of another embodiment of the method of the invention;

FIG. 6D is a diagrammatic illustration of another embodiment of the method of the invention;

FIG. 7A is a diagrammatic illustration of another embodiment of the method of the invention;

FIG. 7B is a diagrammatic illustration of another embodiment of the method of the invention; and

FIG. 8 is a diagrammatic illustration of yet another embodiment of the method of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been discovered that a protein construct formed from a lambdoid bacteriophage gpV protein truncated at its carboxy terminus and peptide linked to a target molecule may successfully be assembled *in vivo* into an infective lambdoid bacteriophage having the target molecule displayed on its outer surface. Furthermore, a phage modified in this manner still retains its ability to infect bacteria. Utilizing such a phage a method of detecting a molecule-of-interest has been developed. In this method, either the death or growth of certain bacterial strains results from the presence of a molecule-of-interest in the solution-to-be-tested depending on the nature of the infecting lambdoid bacteriophage genome and any specific needs of the infected bacteria. This method has also been adapted to select or screen for cell lines that continuously produce a molecule-of-interest.

One type of lambdoid bacteriophage, the bacteriophage lambda, consists of a icosahedral head or capsid with a radius of 30 nm and a flexible tail 150 nm long ending in a tapered basal part and a single tail fiber (FIG. 1A). The genome of the bacteriophage is linear DNA. This DNA is found in the capsid head and has cohesive ends, the right one of which (as defined by the genetic map) protrudes into the upper third of the tail. The tail consists mainly of a tube of 32 disks each consisting of six gpV proteins, the products of the V gene.

In the present invention, a lambdoid bacteriophage is genetically modified so as to expose a target molecule on the outer surface of its tail (FIG. 1B). This is accomplished by providing a truncated gene which encodes at least the amino terminal two-thirds of a lambdoid major tail protein such as, but not limited to, the gpV protein, or other major lambdoid tail protein, and linking this gene fragment to a gene encoding a target protein, thereby forming a gene fusion. The protein product of the gene fusion, i.e., a protein construct, may be expressed in a bacterial cell where it, along with the other phage components, is assembled into a lambdoid bacteriophage if genes encoding the other viral components and enzymes required for phage assembly are present.

The gene fusion may be prepared as follows. The nucleic acid sequence of the V gene is known (Sanger et al. (1982) *J. Mol. Biol.* **162**:729). This gene is simultaneously cloned and modified by PCR methods (Scarf, "Cloning with PCR" in *PCR Protocols. A Guide to Methods and Applications* (Innis et al., eds.) Academic Press, San Diego, CA (1990) pp.84-91), resulting in a full length V gene with its carboxy terminal Ser^{246} codon replaced with a Cys codon TGT. The sequence for the modified V gene is set forth in the Sequence Listing as SEQ ID NO:2. The modified gpV has been cloned into an expression vector (pKK223-3, Pharmacia, Piscataway, NJ) resulting in the pSYM1 plasmid shown in FIG. 4. This plasmid is used to transform *E. coli*. Of course, other plasmids may be used as well. The transformed strain is induced

such as with isopropylthio-ß-D-galactoside (IPTG) (Sambrook et al. in *Molecular Cloning: A Laboratory Manual* (1989) p.17.13. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and then lysed as a source of modified gpV protein. If necessary, the gpV protein can be purified further as described by Katsura et al. (*Virology* (1977) **76**:129). When pSYM1 is digested with PstI (New England Biolabs, Beverly, MA) and religated using T4 DNA ligase (New England Biolabs, Beverly, MA), the pSYM2 plasmid shown in FIG. 5B is obtained. This digestion results in the loss of nucleic acid encoding the C-terminal 24 amino acids of the gpV protein and its replacement by nucleic acid encoding the hexapeptide Ser-Phe-Cys-Phe-Gly-Gly (set forth in the Sequence Listing as SEQ ID NO:7), as depicted in FIG. 3. Of course, the plasmid may be designed such that it may be digested with other restriction endonucleases in the alternative or as well, resulting in the loss of other gpV protein amino acids.

Plasmid pSYM2 has a unique PstI cleavage site near the 3′ terminus of the truncated V gene. The target molecule encoding gene to be fused with the V gene is isolated using the PCR strategy employed for the cloning of the V gene. In this strategy, PCR primers that contain PstI restriction sites are employed to obtain a PstI fragment containing the gene-to-be-fused. This fragment is then ligated to the PstI site in pSYM2 using T4 DNA ligase. This approach requires that there be no PstI site present in the gene-to-be-fused.

When the target molecule gene contains a PstI site, a restriction enzyme that produces blunt ends is used instead of PstI, provided its recognition sequence is not present in the coding region. Such enzymes include BsaAI, Bst1107I, DraI, Ec1136II, Eco47III, EcoRV, and EheI.

After digesting the pSYM2 plasmid with PstI, the PstI site is converted to a blunt end using T4 DNA polymerase. (Maniatis et al., *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982) p.395). Of course, other restriction endonucleases with single recognition sites may be used as well. The gene for the target molecule is then ligated into the plasmid using T4 DNA ligase.

A fragment of another gene encoding at least an antigenic portion of a third protein can be incorporated into the plasmid in a position such as, but not limited to, a position distal to the V gene, resulting in a plasmid such as pSYM3 (FIG. 5C). The third protein may be a marker protein such as ß-galactosidase (encoded by the *lacZ* gene), chloramphenicol acetyltransferase, or alkaline phosphatase, among others. Inclusion of the third protein alleviates the need of obtaining antibodies to the target molecule since antibodies to the third protein can be used to inactivate the phage. Thus, using the same PCR methodology describe above, a V/target/*lacZ* gene fusion can be prepared which encodes a gpV/target molecule/ß-galactosidase or protein construct.

The plasmid pSYM3 (FIG. 5C) is an example of such a plasmid where the gene encoding the target molecule is closed between the V gene and a fragment of *lacZ*. Transcription and translation of this unit results in the production of a gpV/target/ß-galactosidase fusion protein. The plasmid pSYM3 is constructed from pSYM2 as described above where the aforementioned gene-to-be-fused is a 500 bp fragment of *lacZ* beginning at the ATG start codon of the gene. The primer used to anneal to the 5′ end of the *lacZ* gene fragment contains a PstI recognition site, and the primer used for the 3′ end contains a HindIII restriction site. This results in the formation of a V gene/*lacZ* gene fusion which still contains a unique PstI restriction site into which the gene encoding the target molecule can be cloned.

The target molecule can be any protein, polypeptide, or peptide which is translated from a known nucleic acid sequence and which can be peptide bonded to the carboxy terminal end of the truncated gpV protein without abolishing virus assembly or infectivity. Such target molecules include, but are not limited to proteins such as enzymes (e.g., beta-lactamase, triose phosphate isomerase, or hexokinase) enzyme substrates (e.g., pre-interleukin-1, proinsulin, preproinsulin, or erythropoietin) immunoglobulins, or portions thereof (e.g., Fv, Fab, or $(Fab')_2$), receptors or portions thereof (e.g., the estrogen receptor or the insulin receptor), ligands (e.g., ciliary neuronotrophic factor or luteinizing hormone), cytokines (e.g., macrophage migration inhibition factor or the

interleukins), growth factors (e.g., fibroblast growth factor or granulocyte colony stimulating factor) and toxins (e.g., pertussis toxin or botulinum toxin).

When constructing the gene fusion, the process often results in or requires the inclusion of extraneous DNA sequences that, when transcribed and subsequently translated, result in the inclusion of extraneous additional amino acids in the gene fusion product. These additional amino acids may be located between any of the component genes of the construct.

To obtain the modified lambdoid bacteriophage of the invention, the gene fusion encoding the protein construct is provided in a plasmid which is used to transform a bacterial cell such as *E. coli* that can be infected by the bacteriophage. Alternatively, this cell may be preinfected with bacteriophage having nonfunctional gpV protein prior to transformation with the plasmid. The cell is then induced to produce modified phage by chemical stimulation (e.g., with IPTG) and temperature shifting to a high growth temperature (e.g., about 42°C).

The assembled phage are purified from the bacterial cell lysate and then rendered non-infective. This may be accomplished by the binding of a molecule to the target molecule on the bacteriophage. Binding stops the phage from being able to infect a cell. Useful binding molecules

include antibodies or binding portions thereof such as Fv, Fab, or $(Fab')_2$ fragments. The production of such antibodies and biochemically or genetically produced fragments is well known in the art (see, e.g., *Antibodies: A Laboratory Manual* (Harlow and Lane, eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1988).

Other useful binding molecules include receptors which if necessary may be presented in lipid or detergent micelles or liposomes or on cell surfaces to keep their configuration. Such receptor-containing liposomes and micelles can be prepared using any number of methods known in the art (see, e.g., Georgoussi et al. (1990) *Biochem. Biophys. Acta* **1055**:69). When the target molecule is a receptor ligand, the receptor will serve as the immobilizing agent. Receptors which can be presented to the phage in this way include nicotinic acetylcholine receptor (Chak et al. (1992) *Meth. Enzymol.* **207**:546), inositol 1,4,5-triphosphate receptor (Kamata et al. (1992) *J. Biochem.* **111**:546), hepatic vasopressin receptor (Georgoussi, *ibid.*), and the rat ovarian receptor for luteinizing hormone (Kusuda et al. (1986) *J. Biol. Chem.* **261**:16161).

Yet other useful binding molecules include all molecules capable of binding to the target molecule in a competitive fashion. When ligands are used as the binding molecule, they must be immobilized as described in the following paragraph.

Alternatively, the phage can be rendered non-infective by binding it via its target molecule to a matrix. Such matrices include, but are not limited to, commercially available materials such as a gel consisting of dextran cross-linked with epichlorohydrin (e.g., Sephadex™), a special gel prepared from agarose (e.g., Sepharose™), and agarose. When the phage is immobilized to a matrix it is unable to bind to and infect a cell. In this method the phage is immobilized to the matrix and thus is unable to enter and infect a cell. Immobilization to the matrix may be accomplished by chemical linkage or by various chemical cross-linking methods (see, e.g. U.S. Patent No. 5,112,615, herein incorporated by reference, and Wilchek et al. (1984) *Meth. Enzmol.* **104**:3). One type of useful cross-linking reagent is a bifunctional reagent such as β-maleimidopropionic acid N-hydroxysuccinimide ester which can be employed according to the method described in *Laboratory Techniques in Biochemistry and Molecular Biology* (Elsevier Science Publishing Co., Amsterdam, (1988), vol. 19).

The method of the invention has been designed such that the inactivated phage is released or liberated from the matrix or binding molecule by the molecule-of-interest. Thus, if the molecule-of-interest is an enzyme, it can be used to liberate non-infective phage by cleaving target molecule bound to antibodies (FIG. 6A), matrices (FIG. 6B), ligands (FIG. 6C), or receptors (FIG. 6D). In this way, the presence of the molecule-of-interest can be

determined and quantitated by the relative infectivity of the phage.

For example, to detect a molecule-of-interest which is an enzyme capable of cleaving the target molecule (an enzyme substrate), the method of the invention is performed as follows. Expression of the V gene-enzyme substrate gene fusion protein is induced in *E. coli*, carrying either pSYM2 (FIG. 5B) or pSYM3 (FIG. 5C), or another similar V gene-enzyme substrate gene fusion-carrying plasmid; by the addition of 1 mM IPTG (Sambrook et al., in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1989) p. 17.13). The bacteria are then infected with a non-lysogenic lambdoid bacteriophage such as λvir (Arber et al., in *Lambda II* (Hendrix, ed.) Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1983) p. 438). In this case, successful infection results in the production of non-lysogenic lambdoid bacteriophage containing modified gpV protein. The modified bacteriophage are then purified using any purification method known in the art (e.g., Helms et al. (1987) *Meth. Enzymol.* **153**:69-82). The modified bacteriophage are then rendered reversibly non-infective utilizing antibodies directed against either the enzyme substrate (when pSYM2 is employed), or against a marker protein such as ß-galactosidase (Boehringer Mannheim, Indianapolis, IN) (when pSYM3 is employed). Plasmid pSYM3, is preferred because antibodies directed against the marker protein can then be used to inactivate the

bacteriophage regardless of the identity of the target molecule. The desired enzyme present in a solution will cleave the antibody-bound phage-linked enzyme substrate, thereby releasing the phage. The released phage are infective, and thus can be detected by their ability to lyse a cell.

If the molecule-of-interest is a ligand, the method of the invention can be carried out as follows. In this embodiment, expression of the modified V gene-target gene fusion protein is induced in *E. coli* which carries either pSYM2 (FIG. 5B), pSYM3 (FIG. 5C), or some similar V gene-target gene fusion-containing plasmid. Induction can be accomplished by the addition of 1 mM IPTG, as described above, which stimulates the *tac* promoter found in these plasmids. The bacterial cells are then infected with a non-lysogenic lambdoid bacteriophage such as λvir (Arber et al., in *Lambda II* (Hendrix, ed.), Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1983) p. 438). Infection in this case results in cell lysis and the production of non-lysogenic lambdoid bacteriophage containing modified gpV protein. The modified bacteriophage are then isolated as described above and rendered non-infective. This can be accomplished by employing antibodies or binding portions thereof, directed against the target molecule on the outside surface of the bacteriophage. For example, antibodies, when incubated with bacteriophage lambda under the conditions described by Hurwitz et al.

(*Eur. J. Biochem.* (1972) **20**:247-250), cross-link the phages as a result of their divalent nature.

The modified phage may also be rendered non-infective by employing a receptor which binds phage-linked ligand. However, receptors may have to be incorporated into micelles or liposomes as previously noted or presented on the surface of a cell to maintain their configuration for binding ligand (see FIG. 8). Binding of the receptor to the phage-linked ligand adheres the phage to the surface of the micelle, liposome, or cell, thus sterically hindering the ability of the phage to attach to and infect a cell. If the ligand-of-interest is present in the solution-to-be-tested the antibodies (FIG. 7A), or receptor (FIG. 7B) bound to the phage-linked ligand may release the phage in favor of the unbound ligand, thus rendering the phage infective again. Infectivity is measured by screening for cell lysis.

The method of the invention may also be used to detect a cell excreting or secreting a desired ligand, which is the molecule-of-interest (FIG. 8). In this method, a cell that produces the desired ligand (hereafter designated $PopA_1$) is selected from a population (herein designated PopA), that does not produce the ligand. The cells of PopA must be capable of being infected by bacteriophage lambda and must require, for growth, a gene to be supplied by the bacteriophage. For example, a strain of bacteriophage lambda, such as λ *trpE* CIts857, which carries both the temperature sensitive repressor CIts857, and a selectable marker gene, *trpE*

(Frischauf et al. (1983) *J. Mol. Biol.* **170**:827-842), may be employed to infect a bacterial strain carrying the modified gpV protein.

To construct λ *trpE* CIts857, both λEMBL3 DNA and λCIts857 DNA were digested with NheI and the large fragment from λEMBL3 and the small fragment from λCIts857 were isolated by electrophoresis in agarose (Maniatis et al. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 150-170). The isolated fragments were ligated using T4 DNA ligase and the resulting DNA was packaged *in vitro*. The resulting phage were used to infect *E. coli* and a phage stock was prepared from the infected *E. coli* (Davis et al. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratories, Cold Spring, NY pp.74-77).

After IPTG induction of the modified gpV protein, temperature shifting to 42°C results in the production of lambdoid bacteriophage that carry the gene required for growth by all cells present in PopA. Either antibodies directed against the target molecule or a cell receptor specific for the ligand are utilized to render the modified bacteriophage non-infective, as described above. The presence of a ligand-producing bacterial cell $PopA_1$ causes the release of phage by providing unbound ligand to which the phage-linked ligand-bound antibody or receptor can bind instead of the phage-linked ligand. When the antibody or receptor chooses to bind with the unbound ligand, it releases the phage

enabling it to infect the nearby cell which secreted the molecule-of-interest. Infection provides the needed gene, and thereby endows the cell with the ability to grow.

When a temperature sensitive derivative of bacteriophage lambda is employed (e.g., CIts), the ratio of gpV protein to modified gpV protein can be regulated to some extent by varying the time between plasmid (and hence modified gpV protein) expression and bacteriophage (hence gpV protein) expression. Expression of modified gpV is inducible by addition of IPTG. CIts derivatives of bacteriophage lambda are also inducible upon temperature shifting (Maniatis et al., in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982) pp. 78-79). IPTG induction followed by a temperature shift upward to 42°C leads to cell lysis and the release of bacteriophage lambda containing modified gpV.

Likewise, the method of the invention may be used to select a bacterial strain that secretes a molecule-of-interest which is an enzyme from a population that does not secrete the enzyme (FIG. 8). This bacterial strain is auxotrophic for a bacterial component and so will grow only if provided with the component or with a gene capable of correcting the auxotrophy. In this method, a lambdoid bacteriophage that has a temperature sensitive genotype (e.g., CIts 857) and carries a selectable marker gene may be employed to infect a strain carrying a gene fusion encoding gpV protein

modified with an enzyme substrate as the target molecule. After IPTG induction of modified gpV, temperature shifting to 42°C results in cell lysis and the production of bacteriophage lambda carrying the gene required for growth by all cells. Antibodies directed against either the target molecule (when pSYM2 is employed) or ß-galactosidase (when pSYM3 is employed) are used to render the modified bacteriophage non-infective, as described above. Alternatively, the target molecule may be inactivated by immobilization to a matrix or receptor. If an enzyme-producing cell is present, the enzyme produced by the bacterium cleaves the bound, phage-linked target protein, thereby releasing the phage and rendering it infective again. The released phage then infects this auxotrophic cell at low growth temperature, providing it with the gene it needs to survive and grow.

The method of the invention offers several advantages over other systems employing bacteriophages such as M13 or T4. First, any target molecule that can be linked to the gpV protein can be employed as long as it does not completely interfere with *in vivo* assembly or the ability of the resulting bacteriophage to infect bacteria.

Second, the method does not have to result in the death of the infected bacteria. Rather, it can be used to isolate cells that excrete/secrete a desired compound, unlike the M13 and T4 systems. By using a temperature sensitive strain of

bacteriophage lambda and a bacterial cell population that requires for growth a particular gene product supplied by the bacteriophage, those cells that excrete/secrete the desired compound will render infective an inactivated bacteriophage lambda which, in turn, will infect the cell, and at lower temperatures enable the cell to grow. Likewise, the method can be used to isolate either mutant bacterium or a genetically engineered bacterium that excretes or secretes a molecule-of-interest from a population of non-excretors.

Third, this method enables the selective modification of a specific protein, and hence the selective display of a target molecule, unlike the T4 system. With non-specific modifications, a large percentage of the modified phages are rendered permanently non-infective. For example, when nerve growth factor (NGF) was coupled to bacteriophage T4, 76% of the phage were rendered non-infective (Olger et al. (1974) *Proc. Natl. Acad. Sci.* (USA) **71**:1554-1558).

Fourth, as an extension of the method described in the previous paragraph, the method can also be used to screen enzyme libraries for clones having the ability to cleave altered substrate. Immobilization of the bacteriophage via the altered substrate enables isolation of strains from a library that contain an enzyme with the altered specificity from the library. This approach differs from M13 systems where fusion proteins have been used to display proteins because those systems

display only the molecule-of-interest, and thus are not useful for the detection of such molecules. The approach described herein with the lambdoid system is unique in this respect.

The following examples illustrate the preferred mode of making and practicing the present invention, but is not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1. Cloning and Modification of the V Gene

The V gene was simultaneously cloned into the expression vector pkk223-3 (Pharmacia, Piscataway, NJ) and modified using the PCR protocol of Scharf ("Cloning with PCR," in *PCR Protocols. A Guide to Method and Applications* (Innis et al., eds.) Academic Press, San Diego, CA (1990) pp. 84-91). The resulting plasmid is shown in FIG. 5A (pSYM1). The primers used for the procedure are shown in FIG. 4 and are set forth in the Sequence Listing as SEQ ID NOs:3 and 4. The primer that anneals to the 5′ end of the V gene (SEQ ID NO:3) is designed to include an EcoRI restriction endonuclease cleavage site. The primer that anneals to the 3′ end of the V gene (SEQ ID NO:4) is designed to include HindIII and PSTI restriction endonuclease cleavage sites. In addition, this primer contains a single base substitution in the

last codon of the V gene. This substitution results in the conversion of Ser^{246} to Cys^{246}.

The cloned modified V gene is digested with EcoRI and HindIII (New England Biolabs, Beverly, MA) and ligated, using T4 DNA ligase (New England Biolabs, Inc.), into the expression vector pKK223-3 (Pharmacia, Piscataway, NJ) which was digested with EcoRI and HindIII. DNA digestion with the restriction endonucleases, EcoRI and HindIII, was accomplished as described in the New England Biolabs Protocols provided with the endonucleases. The resulting pSYM1, is shown in FIG. 5A.

When pSYM1 is digested with PstI and religated using T4 DNA ligase, the plasmid pSYM2 is obtained (FIG. 5B). This digestion results in the loss of nucleic acid encoding the C-terminal 24 amino acids of the V protein and its replacement by nucleic acid encoding the hexapeptide Ser-Phe-Cys-Phe-Gly-Gly (set forth in the Sequence Listing as SEQ ID NO:7).

The plasmid pSYM3 was formed by replacing the oligonucleotide generated by digesting pSYM2 with PstI and HindIII with a 501 bp fragment of the *E. coli lacZ* gene. The sequence of *lacZ* is available from GenBank (Los Alamos, NM; accession no. J01636). The *lacZ* fragment encodes the first 167 amino acids of the enzyme, ß-galactosidase. The *lacZ* fragment was isolated from λgt11 (Young et al. (1983) *Proc. Natl. Acad. Sci.* USA **80**:1194) using PCR as described above for the isolation of the V gene. The primer that

anneals to the 5′ end of the gene is CCGCTGCAGGAATGACCATGATTACGGATTC (SEQ ID NO:8), wherein the underline sequence is a PstI recognition site and the double underlined sequence is that of the 5′ start of the coding sequence of *lacZ*. The primer that anneals to the 3′ end is CCGAAGCTTAACGACTGTCCTGGCCGTAAC (SEQ ID NO:9), wherein the underlined sequence is a HindIII recognition site and the double underlined sequence is complementary to the 3′ end of the *lacZ* fragment. Both pSYM2 and the PCR-cloned *lacZ* fragment are digested with PstI and HindIII and ligated together using a five-fold molar excess of the *lacZ* fragment. The resulting plasmid, pSYM3, is shown in FIG. 5C.

2. Preparation of Antibody Column

A column having antibodies directed to the target molecule of the V gene protein construct is prepared essentially as described in *Antibodies: A Laboratory Manual* ((Harlow and Lane, eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1988)). Briefly, specific antibodies are mixed with protein A beads (Sigma Chemical Company, St. Louis, MO) using 2 mg of antibody per milliliter of beads. The bead solution is mixed gently for 1 hour at room temperature. The beads are then washed and chemically cross-linked to the antibodies using a bifunctional cross-linking reagent such as dimethylpimelimidate (Sigma Chemical Company). Chemical cross-linking is accomplished by shaking the antibody-coated beads for 30 minutes in the

presence of 20 mM dimethylpimelimidate. The cross-linking reaction is stopped by washing the beads in 0.2 M ethanolamine followed by a 2 hour incubation at room temperature in 0.2 M ethanolamine.

3. Detection of Ciliary Neurotrophic Factor

The gene encoding ciliary neurotrophic factor (CNTF) has been cloned, expressed in chinese hamster ovary (CHO) cells and sequenced (Negro et al. (1991) *Eur. J. Biochem.* 201:289-294). The entire coding sequence for CNTF is also available from Genbank (Los Alamos, NM) (accession no. M29828). This gene does not have any PstI recognition sites. The truncated V gene does contain a Pst site near its 3' terminus: CTGCAG (see SEQ ID NO:1). A PstI fragment containing the CNTF is obtained by PCR using the 5' primer: GTTGCTGCAGGTATGGCTTTCATGGAGCATTCA (SEQ ID NO:5), wherein the underlined sequence is a PstI recognition site and the double underlined sequence is that of the 5' start of the coding sequence for CNTF, and the 3' primer: CTGCAGCTACATTTCCTTGTCGTTAG: (SEQ ID NO:6), wherein the underlined sequence is PstI recognition site and the double underlined sequence is complementary to the 3' end of the coding sequence. Insertion of this PstI fragment into pSYM2 results in the joining of the truncated V gene to the entire CNTF gene. The GT dinucleotide inserted between the PstI recognition site and the beginning of the CNTF coding region is necessary to keep the V gene and CNTF gene in the same open reading frame so that the two genes will be translated into a single polypeptide. This specific

dinucleotide was chosen so as to not introduce any extraneous amino acids into the gene fusion product. Competent *E. coli* SCS1 (Stratagene, La Jolla, CA) is transformed with the resulting plasmid as described by Hanahan (*J. Mol. Biol.* (1983) **166**:557). The transformed strain is induced by IPTG and then incubated with λvir for 15 minutes at 37°C. Top agar is added and the mixture is plated. After 6 hours, the plate is overlayed with lambda dilution buffer (10 mM Tris-HCl, pH 8; 2 mM $MgCl_2$) and incubated overnight at 4°C. Phage containing CNTF are purified from the resulting lysate by running the lysate over an anti-CNTF antibody column, prepared as described above. The CNTF-modified phage are inactivated using anti-CNTF antibodies obtained commercially or by methods well known in the art (see, e.g., *Antibodies: A Laboratory Manual* (Harlow and Lane, eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)). The appropriate ratio of modified phage to antibody is determined experimentally as described by Olger et al. (*Proc. Natl. Acad. Sci.* (USA) (1974) **71**:1554). Inactivated phage are then incubated with media suspected of containing CNTF (the solutions-to-be-tested). The infectivity of the phage are assayed using the plate method of Davis et al. (in *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1980) p.71). The increase in the number of infective phage is directly proportional to the amount of CNTF present in the original sample.

4. Detection of Interleukin-1ß Converting Enzyme

The gene encoding the precursor form of IL-1ß (pIL-1ß) has been cloned and sequenced (March et al. (1985) *Nature* **315**:641-647). The gene encoding pIL-1ß is fused, in frame, to the 3'-terminus of the truncated V gene present in pSYM3, as described above, keeping in mind that the 3' primer must be constructed to produce an "in frame" fusion between the 3' terminus of the pIL-1ß fragment and the 5' terminus of the ß-galactosidase fragment. Competent *E. coli* SCS1 (Stratagene, La Jolla, CA) are transformed with the resulting plasmid. The transformed strain is induced by IPTG and then infected with λvir. Phage containing pIL-1ß are purified from the resulting lysate by running the lysate over the anti-ß-galactosidase antibody column, prepared as described above. The pIL-1ß-modified phage are inactivated using anti-ß-galactosidase antibodies (Boehringer Mannheim, Indianapolis, IN). The appropriate ratio of modified phage to antibody is determined experimentally as described by Olger et al. (*Proc. Natl. Acad. Sci.* (USA) (1974) **71**:1554). Inactivated phage are then incubated with media suspected of containing IL-1ß converting enzyme (ICE), an enzyme which cleaves pIL-1ß to form mature IL-1ß. The infectivity of the phage is then assayed by the plate method of Davis et al. (*ibid.*). The increase in the number of infective phage is directly proportional to the amount of ICE present in the original sample.

5. Selection of Cells Secreting Fibroblast Growth Factor

The gene encoding human fibroblast growth factor (FGF) has been cloned, expressed in *E. coli*, and sequenced (Zazo et al. (1992) *Gene* **113**:231-238). This gene is fused, in frame, to the 3'-terminus of the truncated V gene present in pSYM2, as described above. *E. coli* SCS1 (Stratagene, La Jolla, CA) is transformed with the resulting plasmid, as described above. The transformed strain is induced by IPTG and then infected with λtrpE C1ts857 (Stratagene, La Jolla, CA). Phage containing FGF are purified from the resulting lysate by running the lysate over an anti-FGF antibody column, prepared as described above using commercially obtained anti-FGF antibodies (Sigma Chemical Company, St. Louis, MO). The FGF-modified phage are inactivated using the same anti-FGF antibodies. The appropriate ratio of modified phage to antibody is determined experimentally as described by Olger et al. (*Proc. Natl. Acad. Sci.* (USA) (1974) **71**:1554). Inactivated phage are incubated with *E. coli* Sym3 (having the λ-, F+ ΔtrpE recA hflA genotype) that has been transformed with a mouse brain cDNA library that has been cloned into pYEUra3 (Clontech Laboratories, Palo Alto, CA), and plated on minimal media (*Experiments in Molecular Genetics* (Miller, ed.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1972)) lacking tryptophan. Because *E. coli* Sym3 requires tryptophan for growth, they will grow poorly unless infected by λtrpE C1ts857 which carries a gene that

restores growth of *E. coli* Sym3 on medium lacking tryptophan. Therefore, a cDNA transformant of *E. coli* Sym3 that secretes FGF releases nearby λEMBL3 which then infect the cell resulting in a great enhancement of its growth rate relative to other cells on the plate. The infected cell grows into a visible colony. The colony is then streaked onto the same media, and colonies arising from single cells are those that secrete FGF.

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ray, Bryan L.
Lin, Edmund C.C.
Crea, Roberto

(ii) TITLE OF INVENTION: Method Of Detecting Compounds Utilizing Genetically Modified Lambdoid Bacteriophage

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 200 State Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kerner, Ann-Louise
(B) REGISTRATION NUMBER: 33,523
(C) REFERENCE/DOCKET NUMBER: SYZZ-011PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/330-1300
(B) TELEFAX: 617/330-1311

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 246 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Pro Val Pro Asn Pro Thr Met Pro Val Lys Gly Ala Gly Thr Thr
1 5 10 15

Leu Trp Val Tyr Lys Gly Ser Gly Asp Pro Tyr Ala Asn Pro Leu Ser
20 25 30

Asp Val Asp Trp Ser Arg Leu Ala Lys Val Lys Asp Leu Thr Pro Gly
35 40 45

Glu Leu Thr Ala Glu Ser Tyr Asp Asp Ser Tyr Leu Asp Asp Glu Asp
50 55 60

Ala Asp Trp Thr Ala Thr Gly Gln Gly Gln Lys Ser Ala Gly Asp Thr
65 70 75 80

Ser Phe Thr Leu Ala Trp Met Pro Gly Glu Gln Gly Gln Gln Ala Leu
85 90 95

Leu Ala Trp Phe Asn Glu Gly Asp Thr Arg Ala Tyr Lys Ile Arg Phe
100 105 110

Pro Asn Gly Thr Val Asp Val Phe Arg Gly Trp Val Ser Ser Ile Gly
115 120 125

Lys Ala Val Thr Ala Lys Glu Val Ile Thr Arg Thr Val Lys Val Thr
130 135 140

Asn Val Gly Arg Pro Ser Met Ala Glu Asp Arg Ser Thr Val Thr Ala
145 150 155 160

Ala Thr Gly Met Thr Val Thr Pro Ala Ser Thr Ser Val Val Lys Gly
165 170 175

Gln Ser Thr Thr Leu Thr Val Ala Phe Gln Pro Glu Gly Val Thr Asp
180 185 190

Lys Ser Phe Arg Ala Val Ser Ala Asp Lys Thr Lys Ala Thr Val Ser
195 200 205

Val Ser Gly Met Thr Ile Thr Val Asn Gly Val Ala Ala Gly Lys Val

210 215 220

Asn Ile Pro Val Val Ser Gly Asn Gly Glu Phe Ala Ala Val Ala Glu
225 230 235 240

Ile Thr Val Thr Ala Cys
245

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 741 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGCCTGTAC CAAATCCTAC AATGCCGGTG AAAGGTGCCG GGACCACCCT GTGGGTTTAT 60

AAGGGGAGCG GTGACCCTTA CGCGAATCCG CTTTCAGACG TTGACTGGTC GCGTCTGGCA 120

AAAGTTAAAG ACCTGACGCC CGGCGAACTG ACCGCTGAGT CCTATGACGA CAGCTATCTC 180

GATGATGAAG ATGCAGACTG GACTGCGACC GGGCAGGGGC AGAAATCTGC CGGAGATACC 240

AGCTTCACGC TGGCGTGGAT GCCCGGAGAG CAGGGGCAGC AGGCGCTGCT GGCGTGGTTT 300

AATGAAGGCG ATACCCGTGC CTATAAAATC CGCTTCCCGA ACGGCACGGT CGATGTGTTC 360

CGTGGCTGGG TCAGCAGTAT CGGTAAGGCG GTGACGGCGA AGGAAGTGAT CACCCGCACG 420

GTGAAAGTCA CCAATGTGGG ACGTCCGTCG ATGGCAGAAG ATCGCAGCAC GGTAACAGCG 480

GCAACCGGCA TGACCGTGAC GCCTGCCAGC ACCTCGGTGG TGAAAGGGCA GAGCACCACG 540

CTGACCGTGG CCTTCCAGCC GGAGGGCGTA ACCGACAAGA GCTTTCGTGC GGTGTCTGCG 600

GATAAAACAA AAGCCACCGT GTCGGTCAGT GGTATGACCA TCACCGTGAA CGGCGTTGCT 660

GCAGGCAAGG TCAACATTCC GGTTGTATCC GGTAATGGTG AGTTTGCTGC GGTTGCAGAA 720

ATTACCGTCA CCGCCTGTTA A 741

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc. feature
(B) LOCATION:
(D) OTHER INFORMATION: standardname = "5' Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGGAATTCA ATGCCTGTAC CAAATCCTAC AATG 34

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc. feature
(B) LOCATION:
(D) OTHER INFORMATION: standardname = "3' Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCGAAGCTT CCTGCAGTTA ACAGGCGGTG ACGGTAATTT CTGCAAC 47

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGCTGCAG GTATGGCTTT CATGGAGCAT TCA 33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGCTGCAGC TACATTTCCT TGTCGTTAG 29

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Phe Cys Phe Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc. feature
(B) LOCATION:
(D) OTHER INFORMATION: standardname = "5' Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCGCTGCAGG AATGACCATG ATTACGGATT C 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc. feature
(B) LOCATION:
(D) OTHER INFORMATION: standardname = "3' Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCGAAGCTTA ACGACTGTCC TGGCCGTAAC 30

What is claimed is:

1. A protein construct comprising:

(a) a genetically modified gpV protein truncated at its carboxy terminus; and

(b) a target molecule peptide bonded to the carboxy terminus of the modified gpV protein.

2. The protein construct of claim 1 wherein the target molecule is a protein selected from the group consisting of an enzyme, enzyme substrate, immunoglobulin, toxin, growth factor, cytokine, hormone, ligand, and receptor.

3. The protein construct of claim 1 further comprising at least an antigenic portion of a third protein to which antibodies have been raised.

4. The protein construct of claim 3 wherein the third protein is a marker protein.

5. The protein construct of claim 4 wherein the third protein is a marker protein selected from the group consisting of chloramphenicol acetyltransferase, alkaline phosphatase, and ß-galactosidase.

6. The protein construct of claim 3 wherein the third protein is peptide bonded to the carboxy terminus of the target molecule.

7. A nucleic acid encoding the protein construct of claim 1.

8. A nucleic acid encoding the protein construct of claim 3.

9. A plasmid comprising the nucleic acid of claim 7.

10. A plasmid comprising the nucleic acid of claim 8.

11. An infective lambdoid bacteriophage comprising the protein construct of claim 1, the target molecule being displayed on the outer surface of the bacteriophage.

12. An infective lambdoid bacteriophage comprising the protein construct of claim 3, the target molecule being displayed on the outer surface of the bacteriophage.

13. A method of detecting a molecule-of-interest in a solution comprising the steps of:

(a) providing an infective lambdoid bacteriophage including a protein construct, the protein construct comprising:

(i) a genetically modified gpV protein truncated at its carboxy terminus; and

(ii) a target protein peptide bonded to the carboxy terminus of the gpV protein;

(b) processing the target protein such that the bacteriophage is rendered reversibly non-infective;

(c) treating the non-infective bacteriophage with a solution-to-be-tested, the solution-to-be-tested

containing a molecule-of-interest which renders the non-infective bacteriophage infective;

(d) contacting a bacterial cell with the treated bacteriophage for a time sufficient to enable the bacteriophage to infect the cell; and

(e) detecting bacteriophage infection of the cell, infection being indicative of the presence of the molecule-of-interest in the solution.

14. The method of claim 13 wherein providing step (a) comprises providing an infective lambdoid bacteriophage having a target molecule comprising a protein selected from the group consisting of an enzyme, enzyme substrate, immunoglobulin, receptor, ligand, growth factor, toxin, cytokine, and hormone.

15. The method of claim 13 wherein providing step (a) comprises providing an infective lambdoid bacteriophage including a protein construct, the construct further comprising a peptide linker that is peptide bonded to the carboxy terminus of the gpV protein and to the amino terminus of the target molecule.

16. The method of claim 13 wherein providing step (a) comprises providing an infective lambdoid bacteriophage including a protein construct, the construct further comprising at least an antigenic portion of a third protein.

17. The method of claim 16 wherein providing step (a) comprises providing an infective lambdoid bacteriophage

including a protein construct, the construct comprising at least an antigenic portion of a third protein peptide bonded to the carboxy terminus of the target molecule.

18. The method of claim 13 wherein the providing step (a) comprises:

(i) providing a nucleic acid encoding the protein construct;

(ii) transforming a bacterial cell with the nucleic acid, the cell being pre-infected with a lambdoid bacteriophage assembly mutant having a defective or substantially absent gpV protein;

(iii) inducing the transformed cell to express lambdoid components and to assemble a lambdoid bacteriophage therefrom, the bacteriophage having the target molecule on its outer surface; and

(iv) isolating the lambdoid bacteriophage from the cell.

19. The method of claim 13 wherein the providing step (a) comprises:

(i) providing a lambdoid bacteriophage assembly mutant having a defective or substantially absent gpV protein;

(ii) infecting a bacterial cell with the bacteriophage, the strain being pre-transformed with a nucleic acid encoding the protein construct;

(iii) inducing the infected cell to express lambdoid components and to assemble a lambdoid bacteriophage therefrom having the target molecule on its outer surface; and

(iv) isolating the bacteriophage from the cell.

20. The method of claim 13 wherein processing step (b) comprises treating the bacteriophage with a binding molecule that binds the target molecule, the binding of the target molecule rendering the bacteriophage reversibly non-infective.

21. The method of claim 20 wherein processing step (b) comprises treating the bacteriophage with a binding molecule selected from the group consisting of an enzyme, enzyme substrate, immunoglobulin, receptor, ligand, and matrix.

22. The method of claim 21 wherein processing step (b) comprises immobilizing the bacteriophage-linked target molecule to a matrix.

23. The method of claim 13 wherein treating step (c) comprises treating the non-infective bacteriophage with solution-to-be-tested selected from the group consisting of a culture medium, cell lysate, blood, serum, saliva, semen, and lacrimal secretions.

24. The method of claim 13 wherein treating step (c) comprises treating the non-infective bacteriophage with a molecule-of-interest selected from the group consisting of proteins, peptides, hormones, nucleic acids, carbohydrates, lipids, glycoproteins, glycolipids, proteolipids, lipoproteins, lipopolysaccharides, vitamins, toxins, terpenes, antibiotics, and cofactors.

25. The method of claim 13 wherein treating step (c) comprises treating the non-infective bacteriophage with a solution-to-be-tested, the solution-to-be-tested containing a molecule-of-interest which is an enzyme which cleaves the target molecule.

26. The method of claim 13 wherein treating step (c) comprises treating the non-infective bacteriophage with a solution-to-be-tested, the solution-to-be-tested containing

a molecule-of-interest selected from the group consisting of an unbound target molecule, and an analog, agonist, and antagonist thereof.

27. The method of claim 13 wherein the target molecule and the molecule-of-interest are the same and are ligands, and the binding molecule is a receptor specific for the ligands.

28. The method of claim 13 wherein the target molecule and the molecule-of-interest are the same and are receptors, and the binding molecule is a ligand that binds the receptors.

29. The method of claim 13 wherein the target molecule and the molecule-of-interest contain the same antigenic determinant, and the binding molecule is an immunoglobulin that binds the determinant.

30. The method of claim 13 wherein the target molecule and the molecule-of-interest are the same and are immunoglobulins, and the binding molecule contains an antigenic determinant to which the immunoglobulins bind.

31. The method of claim 13 wherein detecting step (e) comprises detecting cell death, cell death being indicative of the presence in the solution of the molecule-of-interest which has rendered the bacteriophage infective.

32. The method of claim 13 wherein contacting step (d) comprises infecting an auxotrophic bacterial cell with a temperature sensitive bacteriophage at or below about 32°C, the bacteriophage carrying a gene capable of alleviating

the auxotrophy; and detecting step (f) comprises detecting bacterial cell survival and growth, survival and growth being indicative of the presence of the molecule-of-interest in the solution.

33. The method of claim 32 wherein contacting step (d) comprises infecting a bacterial cell incapable of sustained growth with an infective lambdoid bacteriophage lambda carrying a gene capable of restoring sustained growth to the cell; and detecting step (f) comprises detecting bacterial cell survival and growth, survival and growth being indicative of the presence of the molecule-of-interest in the solution.

34. A method of selecting a cell expressing a molecule-of-interest comprising the steps of:

(a) providing an infective lambdoid bacteriophage including a protein construct, the protein construct comprising:

(i) a genetically modified gpV protein truncated at its carboxy terminus; and

(ii) a target protein peptide bonded to the carboxy terminus of the gpV protein;

(b) processing the target protein such that the bacteriophage is rendered reversibly non-infective;

(c) contacting a bacterial cell with the bacteriophage for a time sufficient to enable the molecule-of-interest produced by the cell to render

the non-infective bacteriophage infective and for the inactive bacteriophage to infect the cell; and

(d) detecting bacteriophage infection of the cell, infection being indicative of the presence of the molecule-of-interest in the solution.

35. The method of claim 34 wherein providing step (a) comprises providing a temperature sensitive lambdoid bacteriophage that carries a gene required by the cell to be contacted in step (c); contacting step (c) comprises infecting the cell at or below about 32°C, the cell requiring the gene carried by the bacteriophage for survival and growth; and detecting step (d) comprises detecting the growth of the infected bacteria cell, growth being indicative of the molecule-of-interest in the solution.

36. The method of claim 34 wherein the molecule-of-interest and the target molecule are the same.

37. The method of claim 34 wherein providing step (a) comprises providing a temperature sensitive lambdoid bacteriophage that carried a gene required by the cell to be contacted in step (c) for survival, the gene being selected from the group consisting of a gene required for cell biosynthesis, and a drug resistance gene.

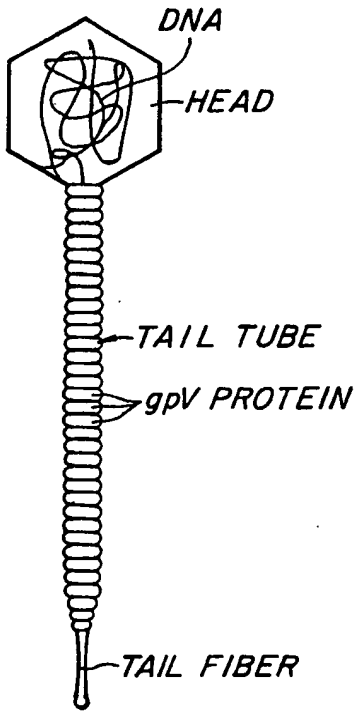

FIG. 1A

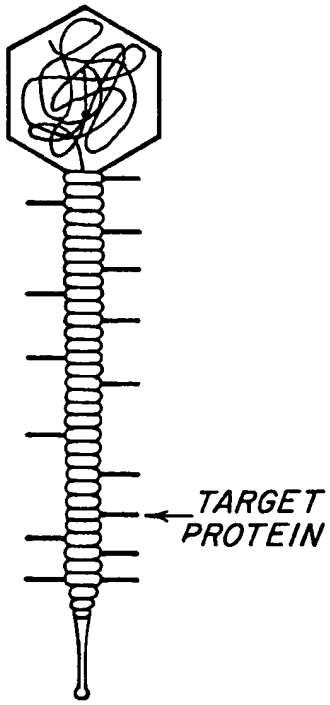

FIG. 1B

```
                                M  F  V  F  N  F  T  M  P  V  K
                                ATGCCTGTACCAAATCCTACAATGCCGGTGAA

  G  A  G  T  T  L  W  V  Y  K  G  S  G  D  P  Y
AGGTGCCGGCACCACCCTGTGGGTTTATAAGGGGAGCGGTGACCCTTACG

A  N  P  L  S  D  V  D  W  S  R  L  A  K  V  K  D
CGAATCCGCTTTCAGACGTTGACTCGTCGCGTCTGGCAAAAGTTAAAGAC

  L  T  P  G  E  L  T  A  E  S  Y  D  D  S  Y  L  D
CTGACGCCCGGCGAACTGACCGCTGAGTCCTATGACGACAGCTATCTCGA

  D  E  D  A  D  W  T  A  T  G  Q  G  Q  K  S  A
TGATGAAGATGCAGACTGGACTGCGACCGGGCAGGGGCAGAAATCTGCCG

G  D  T  S  F  T  L  A  W  M  P  G  E  Q  G  Q  Q
GAGATACCAGCTTCACGCTGGCGTGGATGCCCGGAGAGCAGGGGCAGCAG

  A  L  L  A  W  F  N  E  G  D  T  R  A  Y  K  I  R
GCGCTGCTGGCGTGGTTTAATGAAGGCGATACCCGTGCCTATAAAATCCG

  F  P  N  G  T  V  D  V  F  R  G  W  V  S  S  I
CTTCCCGAACGGCACGGCACATGTGTTCCGTGGCTGGGTCAGCAGTATCG

G  K  A  V  T  A  K  E  V  I  T  R  T  V  K  V  T
GTAAGGCGGTGACGGCGAAGGAAGTGATCACCCGCACGGTGAAAGTCACC

  N  V  G  R  P  S  M  A  E  D  R  S  T  V  T  A  A
AATGTGGGACGTCCGTCGATGGCAGAAGATCGCAGCACGGTAACAGCGGC

  T  G  M  T  V  T  P  A  S  T  S  V  V  K  G  Q
AACCGGCATGACCGTGACGCCTGCCAGCACCTCGGTGGTGAAAGGGCAGA

S  T  T  L  T  V  A  F  Q  P  E  G  V  T  D  K  S
GCACCACGCTGACCGTGGCCTTCCAGCCGGAGGGCGTAACCGACAAGAGC

  F  R  A  V  S  A  D  K  T  K  A  T  V  S  V  S  G
TTTCGTGCGGTGTCTGCGGATAAAACAAAAGCCACCGTGTCGGTCAGTGG

  M  T  I  T  V  N  G  V  A  A  G  K  V  N  I  P
TATGACCATCACCGTGAACGGCGTTGCTGCAGGCAAGGTCAACATTCCGG

V  V  S  G  N  G  E  F  A  A  V  A  E  I  T  V  T
TTGTATCCGGTAATGGTGAGTTTGCTGCGGTTGCAGAAATTACCGTCACC

  A  S
GCCAGT
```

FIG. 2

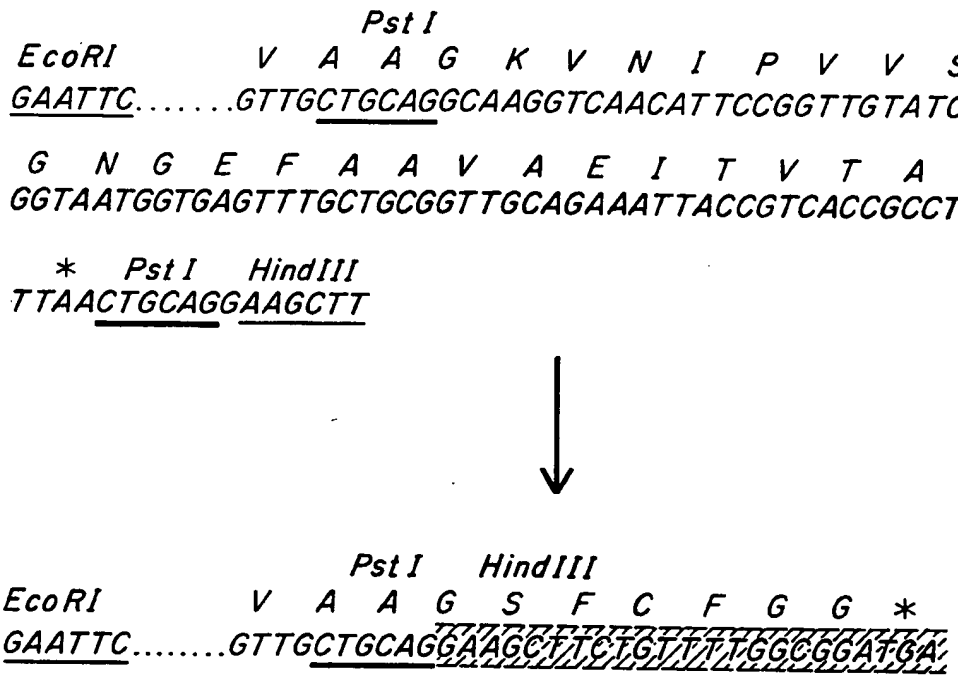
Pst I
EcoRI V A A G K V N I P V V S
GAATTC.....GTTGCTGCAGGCAAGGTCAACATTCCGGTTGTATCC
G N G E F A A V A E I T V T A G
GGTAATGGTGAGTTTGCTGCGGTTGCAGAAATTACCGTCACCGCCTG
* Pst I HindIII
TTAACTGCAGGAAGCTT
Pst I HindIII
EcoRI V A A G S F C F G G *
GAATTC.....GTTGCTGCAGGAAGCTTCTGTTTTGGCGGATGA

FIG. 3

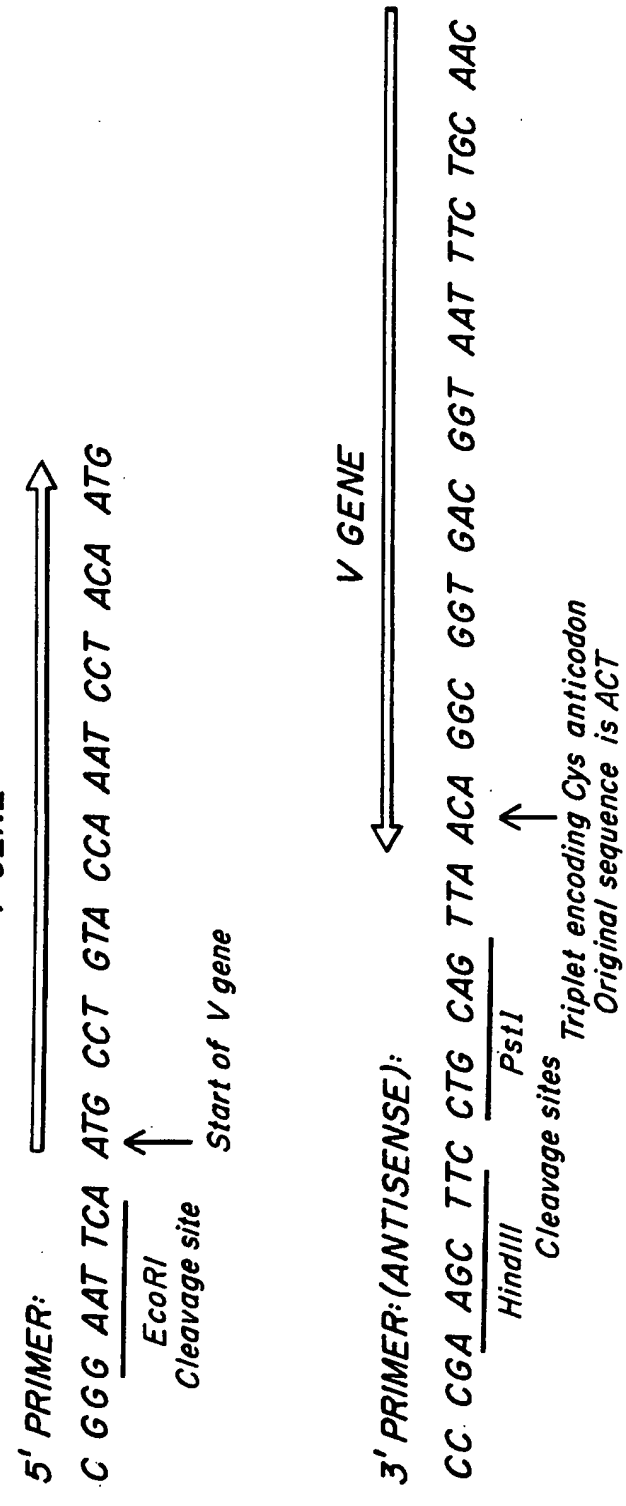
V GENE
5' PRIMER:
C GGG AAT TCA ATG CCT GTA CCA AAT CCT ACA ATG
EcoRI
Cleavage site
Start of V gene
V GENE
3' PRIMER: (ANTISENSE):
CC CGA AGC TTC CTG CAG TTA ACA GGC GGT GAC GGT AAT TTC TGC AAC
HindIII
PstI
Cleavage sites
Triplet encoding Cys anticodon
Original sequence is ACT

FIG. 4

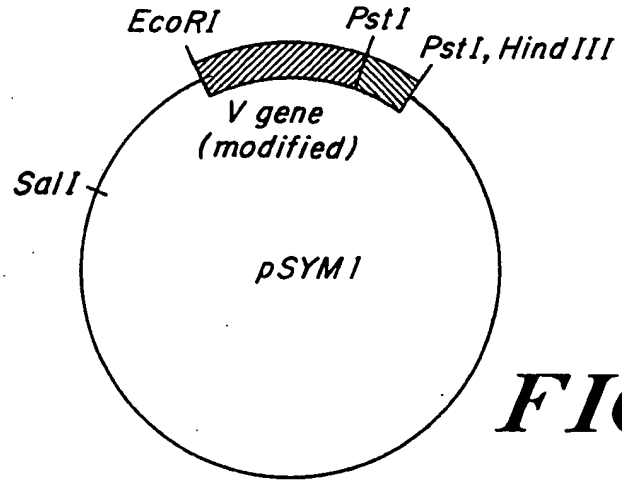

FIG. 5A

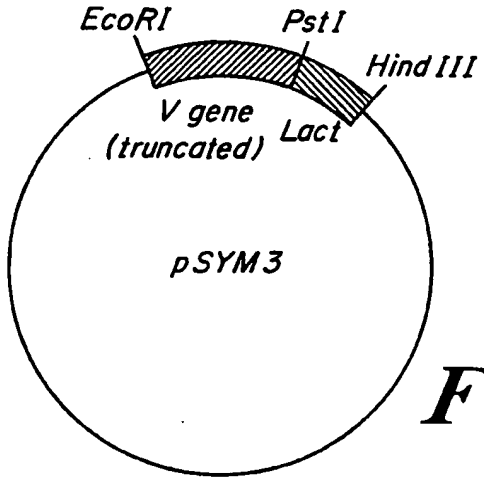

FIG. 5C

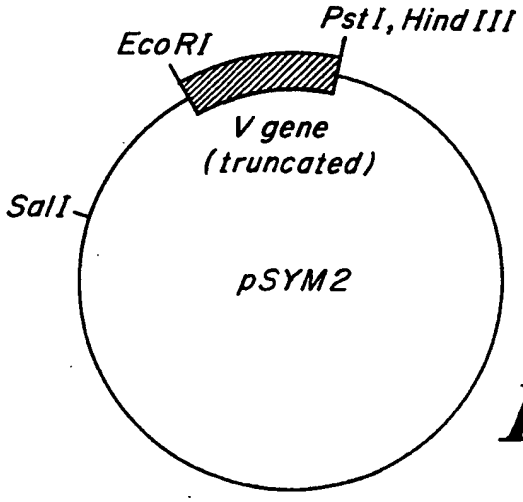

FIG. 5B

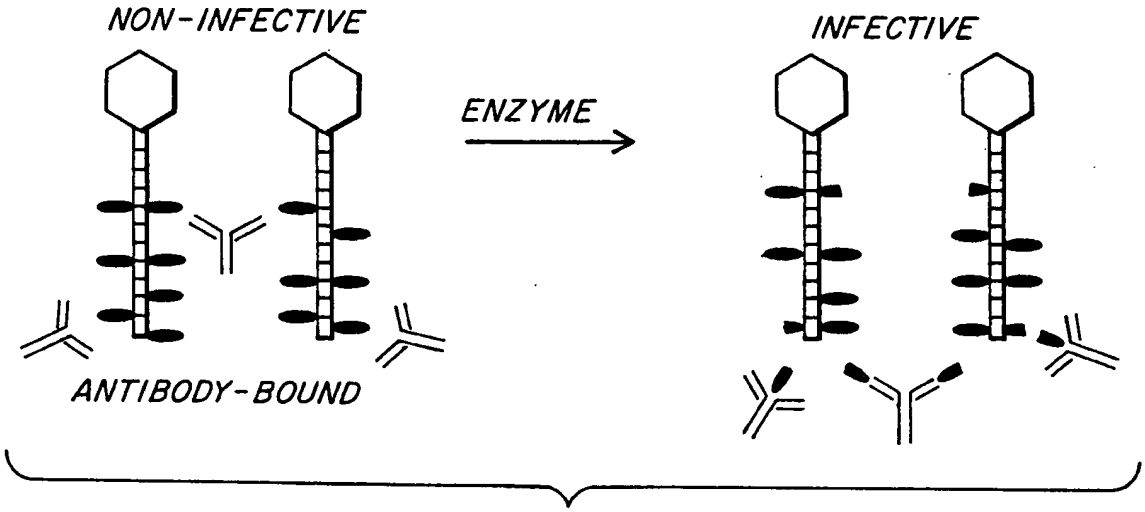
NON-INFECTIVE
ENZYME
INFECTIVE
ANTIBODY-BOUND

FIG. 6A

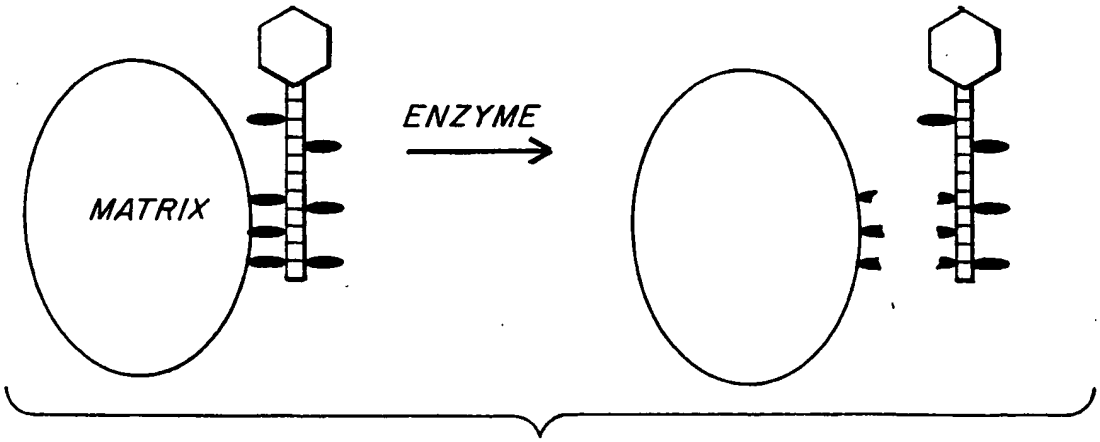
MATRIX
ENZYME

FIG. 6B

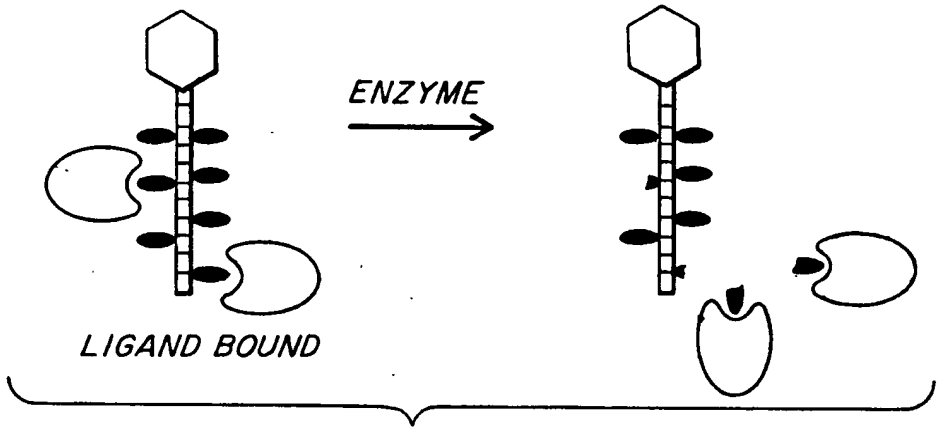

FIG. 6C

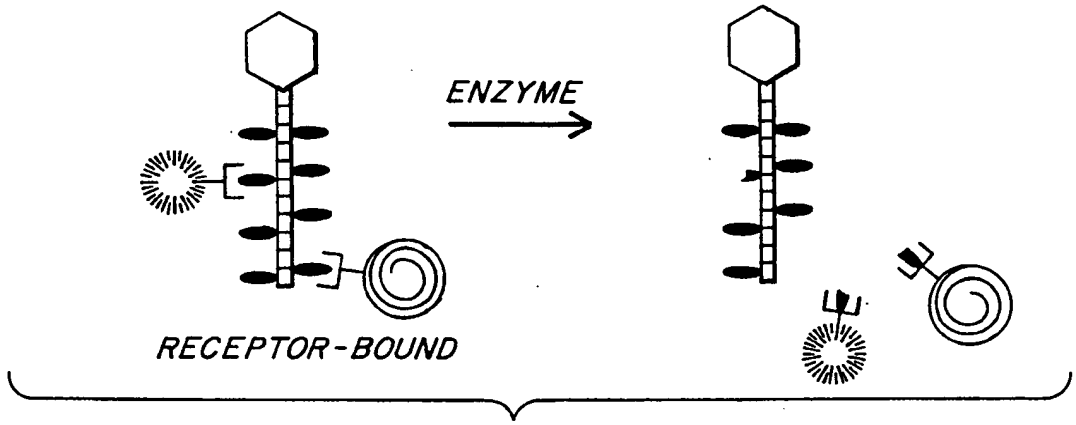

FIG. 6D

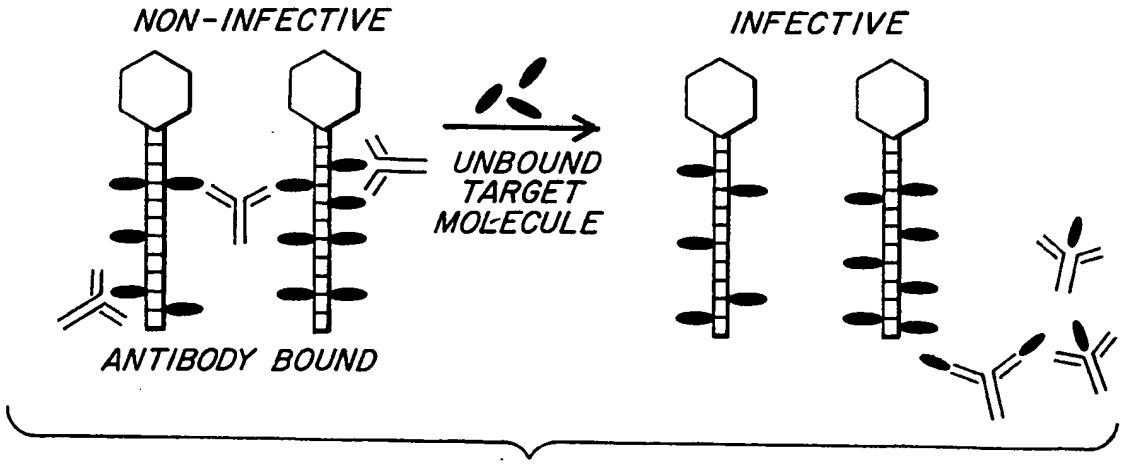
NON-INFECTIVE
INFECTIVE
UNBOUND TARGET MOLECULE
ANTIBODY BOUND

FIG. 7A

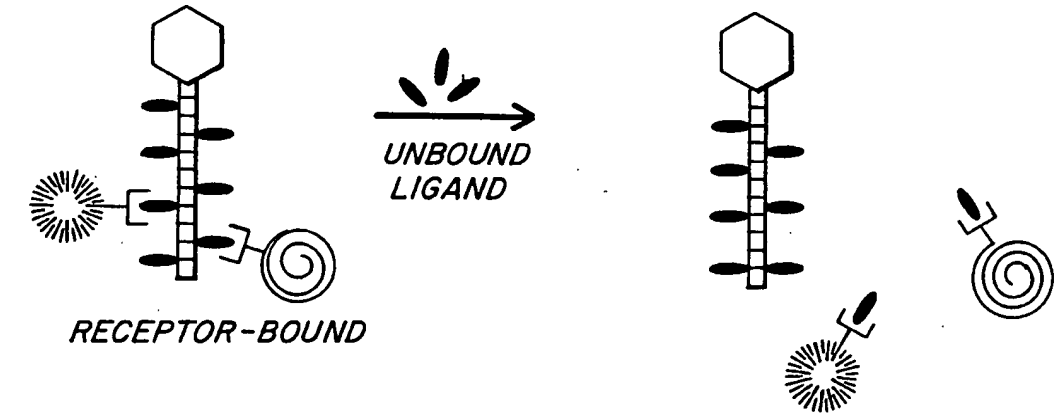
UNBOUND LIGAND
RECEPTOR-BOUND

FIG. 7B

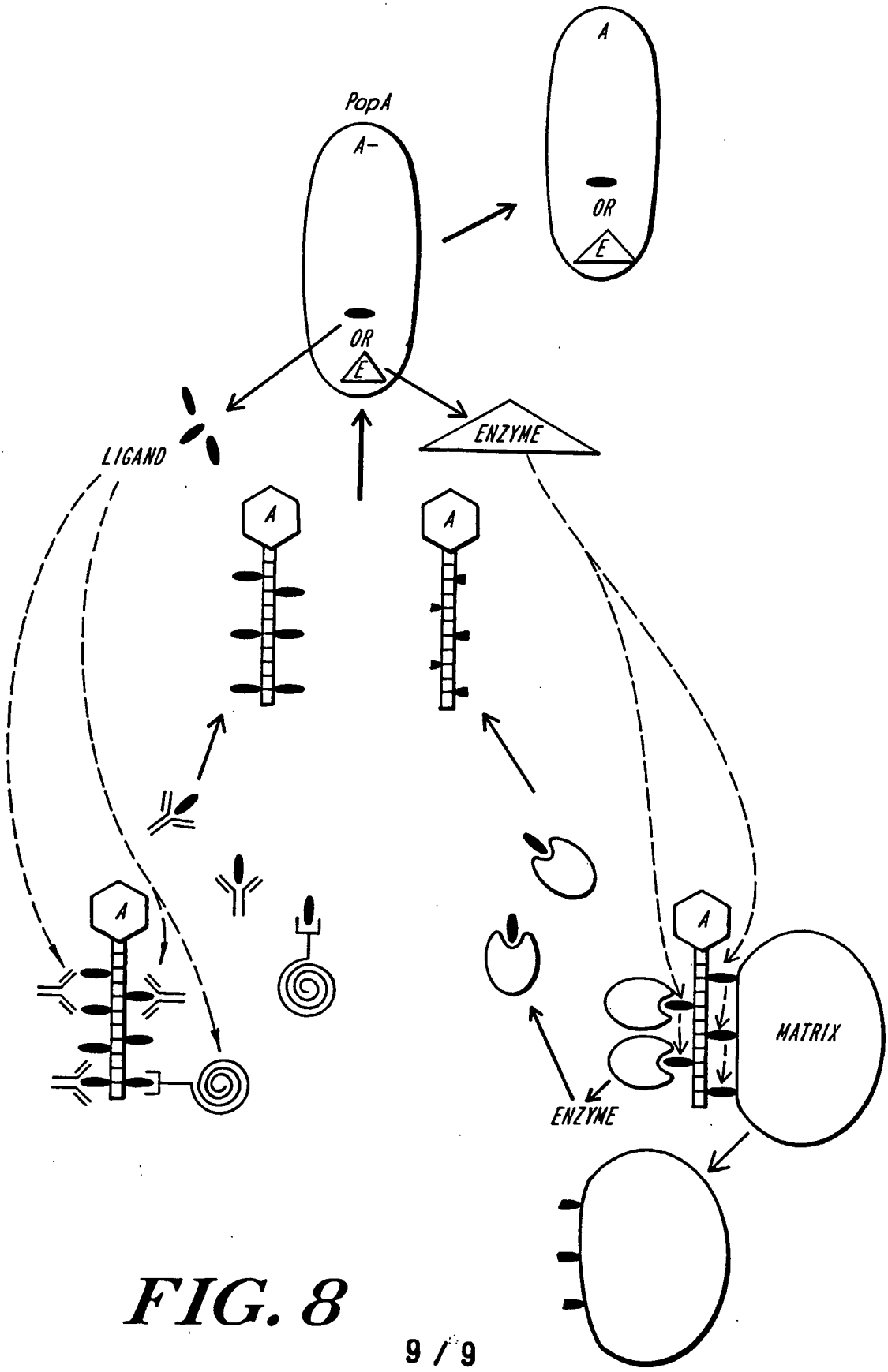

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06543

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/70, 1/68, 1/02; C12N 15/70, C07K 15/00
US CL :435/6, 5, 172.3, 320.1; 536/23.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 7.1, 7.32, 7.37, 7.4, 172.3, 320.1; 536/23.1, 23.2, 23.4, 23.53; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 249, issued 27 July 1990, Devlin et al., "Random Peptide Libraries: A Source of Specific Protein Binding Molecules", pages 404-406, see entire document.	1-37
Y	Science, Volume 249, issued 27 July 1990, Scott et al., "Searching for Peptide Ligands with an Epitope Library", pages 386-390, see entire document.	1-37
Y	BIO/TECHNOLOGY, Volume 9, issued December 1991, Garrard et al., "F_{ab} Assembly And Enrichment In A Monovalent Phage Display System", pages 1373-1377, see entire document.	1-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
22 AUGUST 1994	SEP 02 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROBERT A. HODGES Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 88, issued September 1991, Barbas et al., "Assembly of combinatorial antibody libraries on phage surfaces: The gene III site", pages 7978-7982, see entire document.	1-37
Y	Journal of Molecular Biology, Volume 146, issued 1981, I. Katsura, "Structure and Function of the Major Tail Protein of Bacteriophage Lambda", pages 493-512, see especially the abstract.	1-37
Y	European Journal of Biochemistry, Volume 26, Number 2, issued 1972, Gurari et al., "Use of Immunologically Modified Bacteriophage T4 in Detection of Antibodies to Nucleic Acids", pages 247-250, see especially pages 248 and 249.	13-37
Y	European Journal of Biochemistry, Volume 17, Number 2, issued 1970, Hurwitz et al., "A Sensitive Technique for Detecting and Estimating the Peptide Hormone Angiotensin-II-ß-amide and its Antibodies by Using Chemically Modified Bacteriophage and Activated Sepharose", pages 273-277, see especially page 276.	13-37
Y	Immunochemistry, Volume 7, issued 1970, Becker et al., "Detection of anti-*p*-azobenzenearsonate antibodies with chemically modified bacteriophage", pages 741-743, see entire document.	13-37
Y	Proceedings of the National Academy of Sciences USA, Volume 71, Number 4, issued April 1974, Oger et al., "Synthesis of Nerve Growth Factor by L and 3T3 Cells in Culture", pages 1554-1558, see page 1555.	13-37